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(54) Sigma subunits of Mycobacterium tuberculosis RNA polymerase

(57) The present invention provides novel nucleic acid molecules coding for sigma subunits of Mycobacterium tuberculosis RNA polymerase. It also relates to polypeptides, referred to as SigA and SigB, encoded by such nucleic acid molecules, as well as to vectors and host cells transformed with the said nucleic acid molecules. The invention further provides screening assays for compounds which inhibit the interaction between a sigma subunit and a core RNA polymerase.

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Fig. 1

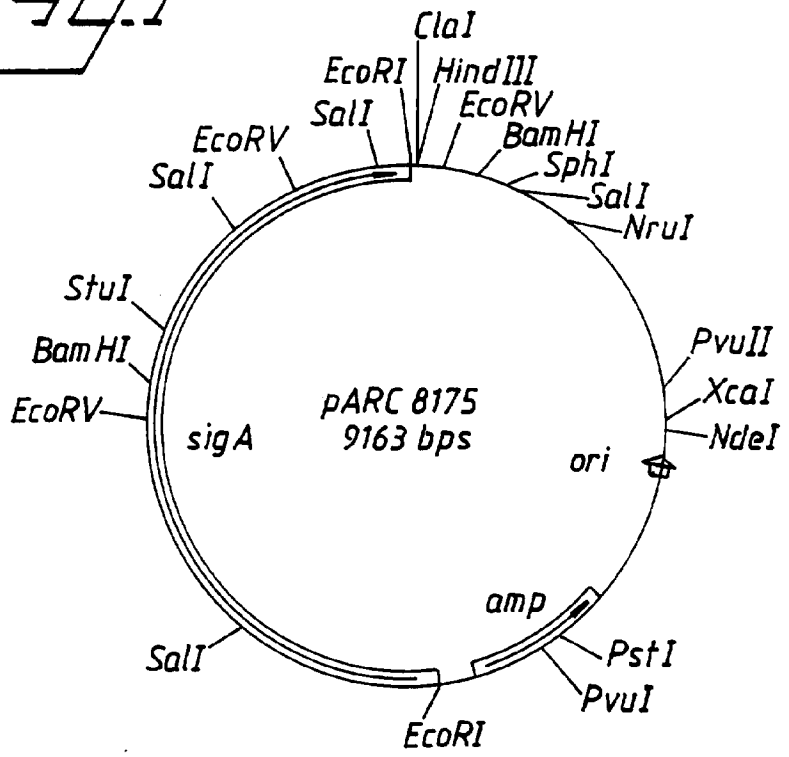
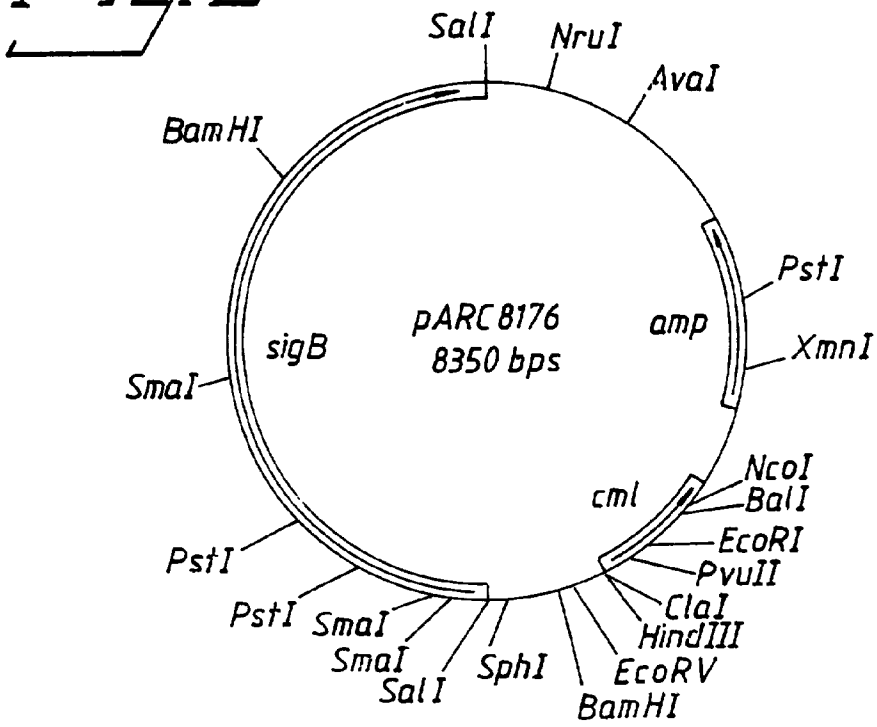

ClaI
EcoRI
HindIII
SalI
EcoRV
BamHI
EcoRV
SphI
SalI
SalI
NruI
StuI
PvuII
BamHI
XcaI
EcoRV
pARC 8175
9163 bps
NdeI
sigA
ori
amp
SalI
PstI
PvuI
EcoRI

Fig. 2


SalI
NruI
AvaI
BamHI
PstI
pARC 8176
8350 bps
amp
sigB
XmnI
SmaI
NcoI
cml
BalI
EcoRI
PstI
PvuII
ClaI
PstI
SmaI
HindIII
SmaI
SphI
EcoRV
SalI
BamHI

NEW DNA MOLECULES

2298862

TECHNICAL FIELD

The present invention provides novel nucleic acid molecules coding for sigma subunits of *Mycobacterium tuberculosis* RNA polymerase. It also relates to polypeptides, referred to as SigA and SigB, encoded by such nucleic acid molecules, as well as to vectors and host cells transformed with the said nucleic acid molecules. The invention further provides screening assays for compounds which inhibit the interaction between a sigma subunit and a core RNA polymerase.

BACKGROUND ART

Transcription of genes to the corresponding RNA molecules is a complex process which is catalyzed by DNA dependent RNA polymerase, and involves many different protein factors. In eubacteria, the core RNA polymerase is composed of α, β, and β′ subunits in the ratio 2:1:1. To direct RNA polymerase to promoters of specific genes to be transcribed, bacteria produce a variety of proteins, known as sigma (σ) factors, which interact with RNA polymerase to form an active holoenzyme. The resulting complexes are able to recognize and attach to selected nucleotide sequences in promoters.

Physical measurements have shown that the sigma subunit induces conformational transition upon binding to the core RNA polymerase. Binding of the sigma subunit to the core enzyme increases the binding constant of the core enzyme for DNA by several orders of magnitude (Chamberlin, M.J. (1974) Ann. Rev. Biochem. 43, 721-).

Characterisation of sigma subunits, identified and sequenced from various organisms, allows them to be classified into two broad categories; Group I and Group II. The Group I sigma has also been referred to as the sigma^{70} class, or the "house keeping" sigma group. Sigma subunits belonging to this group recognise similar promoter sequences in the cell. These properties are reflected in certain regions of the proteins which are highly conserved between species.

Bacterial sigma factors do not have any homology with eukaryotic transcription factors, and are consequently a potential target for antibacterial compounds. Mutations in the sigma subunit, effecting its association and ability to confer DNA sequence specificity to the enzyme, are known to be lethal to the cell.

Mycobacterium tuberculosis is a major pulmonary pathogen which is characterized by its very slow growth rate. As a pathogen it gains access to alveolar macrophages where it multiplies within the phagosome, finally lysing the cells and being disseminated through the blood stream, not only to other areas of the lung, but also to extrapulmonary tissues. Thus the pathogen multiplies in at least two entirely different environments, which would involve the utilisation of different nutrients and a variety of possible host factors; a successful infection would thus involve the coordinated expression of new sets of genes. This regulation would resemble different physiological stages, as best exemplified by *Bacillus*, in which the expression of genes specific for different stages are transcribed by RNA polymerases associating with different sigma factors. This provides the possibility of targeting not only the house keeping sigma of *M. tuberculosis*, but also sigma subunits specific for the different stages of infection and dissemination.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Map of plasmid pARC 8175

Fig. 2: Map of plasmid pARC 8176

PURPOSE OF THE INVENTION

Since the association to a specific sigma subunit is essential for the specificity of RNA polymerase, this process of association is a suitable target for drug design. In order to identify compounds capable of inhibiting the said association process, the identification of the primary structures of sigma subunits is desirable.

It is thus the purpose of the invention to provide information on sequences and structure of sigma subunits, which information will enable the screening, identification and design of compounds competing with the sigma subunit for binding to the core RNA polymerase, which compounds may be developed into effective therapeutic agents.

DISCLOSURE OF THE INVENTION

Throughout this description and in particular in the following examples, the terms "standard protocols" and "standard procedures", when used in the context of molecular cloning techniques, are to be understood as protocols and procedures found in an ordinary laboratory manual such as: Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

In a first aspect, this invention provides an isolated polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* RNA polymerase, or a functionally equivalent modified form thereof.

Preferred such polypeptides having amino acid sequences according to SEQ ID NO: 2 or 4 of the Sequence Listing have been obtained by recombinant DNA techniques and are hereinafter referred to as SigA and SigB polypeptides. However, it will be understood that the polypeptides according to the invention are not limited strictly to polypeptides with an amino acid sequence identical with SEQ ID NO: 2 or 4 in the Sequence Listing. Rather the invention additionally encompasses modified forms of these native polypeptides carrying modifications like substitutions, small deletions, insertions or inversions, which polypeptides nevertheless have substantially the biological activities of a *M. tuberculosis* sigma subunit. Such biological activities comprise the ability to associate with the core enzyme and / or confer the property of promoter sequence recognition and initiation of transcription. Included in the invention are consequently polypeptides, the amino acid sequence of which are at least 90% homologous, preferably at least 95% homologous, with the amino acid sequence shown as SEQ ID NO: 2 or 4 in the Sequence Listing.

In another aspect, the invention provides isolated and purified nucleic acid molecules which have a nucleotide sequence coding for a polypeptide of the invention e.g. the SigA or SigB polypeptide. In a preferred form of the invention, the said nucleic acid molecules are DNA molecules which have a nucleotide sequence identical with SEQ ID NO: 1 or 3 of the Sequence Listing. However, the nucleic acid molecules according to the invention are not to be limited strictly to the DNA molecules with the sequence shown as SEQ ID NO: 1 or 3. Rather the invention encompasses nucleic acid molecules carrying modifications like substitutions, small deletions, insertions or inversions, which nevertheless encode proteins having substantially the biochemical activity of the polypeptides according to the

invention. Included in the invention are consequently DNA molecules, the nucleotide sequences of which are at least 90% homologous, preferably at least 95% homologous, with the nucleotide sequence shown as SEQ ID NO: 1 or 3 in the Sequence Listing.

Included in the invention are also DNA molecule which nucleotide sequences are degenerate, because of the genetic code, to the nucleotide sequences shown as SEQ ID NO: 1 or 3. A sequential grouping of three nucleotides, a "codon", codes for one amino acid. Since there are 64 possible codons, but only 20 natural amino acids, most amino acids are coded for by more than one codon. This natural "degeneracy", or "redundancy", of the genetic code is well known in the art. It will thus be appreciated that the DNA sequence shown in the Sequence Listing is only an example within a large but definite group of DNA sequences which will encode the polypeptide as described above.

Included in the invention are consequently isolated nucleic acid molecule selected from:

(a) DNA molecules comprising a nucleotide sequence as shown in SEQ ID NO: 1 or SEQ ID NO: 3 encoding a Group I sigma subunit of *Mycobacterium tuberculosis* RNA polymerase;

(b) nucleic acid molecules comprising a nucleotide sequence capable of hybridizing to a nucleotide sequence complementary the polypeptide coding region of a DNA molecule as defined in (a) and which codes for a polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* or a functionally equivalent modified form thereof; and

(c) nucleic acid molecules comprising a nucleic acid sequence which is degenerate, as a result of the genetic code, to a nucleotide sequence as defined in (a) or (b) and which codes for a polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* or a functionally equivalent modified form thereof.

The term "hybridizing to a nucleotide sequence" should be understood as hybridizing to a nucleotide sequence, or a specific part thereof, under stringent hybridization conditions which are known to a person skilled in the art.

A DNA molecule of the invention may be in the form of a vector, e.g. a replicable expression vector which carries and is capable of mediating the expression of a DNA molecule according to the invention. In the present context the term "replicable" means that the vector is able to replicate in a given type of host cell into which is has been introduced. Examples of vectors are viruses such as bacteriophages, cosmids, plasmids and other recombination vectors. Nucleic acid molecules are inserted into vector genomes by methods well known in the art. Vectors according to the invention can include the plasmid vector pARC 8175 (NCIMB 40738) which contains the coding sequence of the *sigA* gene, or pARC 8176 (NCIMB 40739) which contains the coding sequence of the *sigB* gene.

Included in the invention is also a host cell harbouring a vector according to the invention. Such a host cell can be a prokaryotic cell, a unicellular eukaryotic cell or a cell derived from a multicellular organism. The host cell can thus e.g. be a bacterial cell such as an *E. coli* cell; a cell from a yeast such as *Saccharomyces cervisiae* or *Pichia pastoris*, or a mammalian cell. The methods employed to effect introduction of the vector into the host cell are standard methods well known to a person familiar with recombinant DNA methods.

A further aspect of the invention is a process for production of a polypeptide of the invention, comprising culturing host cells transformed with an expression vector according of the invention under conditions whereby said polypeptide is produced, and recovering said polypeptide.

The medium used to grow the cells may be any conventional medium suitable for the purpose. A suitable vector may be any of the vectors described above, and an appropriate host cell may be any of the cell types listed above. The methods employed to construct the vector and effect introduction thereof into the host cell may be any methods known for such purposes within the field of recombinant DNA. The recombinant polypeptide expressed by the cells may be secreted, i.e. exported through the cell membrane, dependent on the type of cell and the composition of the vector.

If the polypeptide is produced intracellularly by the recombinant host, i.e. is not secreted by the cell, it may be recovered by standard procedures comprising cell disrupture by mechanical means, e.g. sonication or homogenization, or by enzymatic or chemical means followed by purification.

In order to be secreted, the DNA sequence encoding the polypeptide should be preceded by a sequence coding for a signal peptide, the presence of which ensures secretion of the polypeptide from the cells so that at least a significant proportion of the polypeptide expressed is secreted into the culture medium and recovered.

Another important aspect of the invention is a method of assaying for compounds which have the ability to inhibit the association of a sigma subunit to a *Mycobacterium tuberculosis* RNA polymerase, said method comprising the use of a recombinant SigA or SigB polypeptide or a nucleic acid molecule as defined above. Such a method will preferably comprise (i) contacting a compound to be tested for such inhibition ability with a SigA or SigB polypeptide as described above and a *Mycobacterium tuberculosis* core RNA polymerase; and (ii) detecting whether the said polypeptide associates with the said core RNA polymerase to form RNA polymerase holoenzyme. The term "core RNA polymerase" is to be understood as an

RNA polymerase which comprises at least the α, β, and β' subunits, but not the sigma subunit. The term "RNA polymerase holoenzyme" is to be understood as an RNA polymerase comprising at least the α, β, β' and sigma subunits. If desirable, the sigma subunit polypeptide can be labelled, for example with a suitable radioactive molecule, e.g. ^{35}S or ^{125}I.

Suitable methods for determining whether a sigma polypeptide has associated to core RNA polymerase are disclosed by Lesley et al. (Biochemistry 28, 7728-7734, 1989). Such a method may thus be based on the size difference between sigma polypeptides bound to core RNA polymerase, versus polypeptides not bound. This difference in size allows the two forms to be separated by chromatography, e.g. on a gel filtration column, such as a Waters Protein Pak® 300SW sizing column. The two forms eluted from the column may be detected and quantified by known methods, such as scintillation counting or SDS-PAGE followed by immunoblotting.

According to another method also described by Lesley et al. *(supra)*, RNA polymerase holoenzyme is detected by immunoprecipitation using an antibody binding to RNA polymerase holoenzyme. Core RNA polymerase from an organism such as *E. coli*, *M. tuberculosis* or *M. smegmatis* can be allowed to react with a radiolabelled SigA or SigB polypeptide. The reaction mix is treated with *Staphylococcus aureus* formalin-treated cell suspension, pretreated with an anti-RNA polymerase antibody. The cell suspension is washed to remove unbound proteins, resuspended in SDS-PAGE sample buffer and separated on SDS-PAGE. Bound SigA or SigB polypeptides are monitored by autoradiography followed by scintillation counting.

Another method of assaying for compounds which have the ability to inhibit sigma subunit-dependent transcription by a *Mycobacterium tuberculosis* RNA polymerase can comprise (i) contacting a compound to be

tested for said inhibition ability with a polypeptide of the invention, a *Mycobacterium tuberculosis* core RNA polymerase, and a DNA having a coding sequence operably-linked to a promoter sequence capable of recognition by said core RNA polymerase when bound to said polypeptide, said contacting being carried out under conditions suitable for transcription of said coding sequence when *Mycobacterium tuberculosis* RNA polymerase is bound to said promoter; and (ii) detecting formation of mRNA corresponding to said coding sequence.

Such an assay is based on the fact that *E. coli* consensus promoter sequences are not transcribable by core RNA polymerase lacking the sigma subunit. However, addition of a sigma^{70} protein will enable the complex to recognise specific promoters and initiate transcription. Screening of compounds which have the ability to inhibit sigma-dependent transcription can thus be performed, using DNA containing a suitable promoter as a template, by monitoring the formation of mRNA of specific lengths. Transcription can be monitored by measuring incorporation of ^{3}H-UTP into TCA-precipitable counts (Ashok Kumar et al. (1994) J. Mol. Biol. 235, 405-413; Kajitani, M. and Ishihama, A. (1983) Nucleic Acids Res. 11, 671-686 and 3873-3888) and determining the length of the specific transcript. Compounds which are identified by such an assay can inhibit transcription by various mechanisms, such as (a) binding to a sigma protein and preventing its association with the core RNA polymerase; (b) binding to core RNA polymerase and sterically inhibiting the binding of a sigma protein; or (c) inhibiting intermediate steps involved in the initiation or elongation during transcription.

A further aspect of the invention is a method of determining the protein structure of a *Mycobacterium tuberculosis* RNA polymerase sigma subunit, characterised in that a SigA or SigB polypeptide is utilized in X-ray crystallography. The use of SigA or SigB polypeptide in crystallisation will facilitate a rational design, based on X-ray crystallography, of therapeutic

compounds inhibiting interaction of a sigma^{70} protein with the core RNA polymerase, alternatively inhibiting the binding of a sigma70 protein, in association with a core RNA polymerase, to DNA during the course of gene transcription.

EXAMPLES

EXAMPLE 1: Identification of *M. tuberculosis* DNA sequences homologous to the sigma^{70} gene

1.1. PCR amplification of putative sigma^{70} homologues

The following PCR primers were designed, based on the conserved amino acid sequences of sigma^{45} (a sigma^{70} homologue) of *Bacillus subtilis* and sigma^{70} of *E. coli* (Gitt, M.A. et al. (1985) J. Biol. Chem. 260, 7178-7185):

Forward primer (SEQ ID NO: 5):

```
5'-AAG TTC AGC ACG TAC GCC ACG TGG TGG ATC-3'
                    C       G   C
```

Reverse primer (SEQ ID NO: 6):

```
5'-CTT GGC CTC GAT CTG GCG GAT GCG CTC-3.
       C                C       C
```

The alternative nucleotides indicated at certain positions indicate that the primers are degenerate primers suitable for amplification of the unidentified gene.

Chromosomal DNA from *M. tuberculosis* H37RV (ATCC 27294) was prepared following standard protocols. PCR amplification of a DNA fragment of approximately 500 bp was carried out using the following conditions:

Annealing:	+55°C	1 min
Denaturation:	+93°C	1 min
Extension:	+73°C	2 min

1.2. Southern hybridisation of M. tuberculosis DNA

Chromosomal DNA from *M. tuberculosis* H37RV (ATCC 27294), *M. tuberculosis* H37RA and *Mycobacterium smegmatis* was prepared following standard protocols and restricted with the restriction enzyme *Sal*I. The DNA fragments were resolved on a 1% agarose gel by electrophoresis and transferred onto nylon membranes which were subjected to "Southern blotting" analysis following standard procedures. To detect homologous fragments, the membranes were probed with a radioactively labelled ~500 bp DNA fragment, generated by PCR as described above.

Analysis of the Southern hybridisation experiment revealed the presence of at least three hybridising fragments of approximately 4.2, 2.2 and 0.9 kb, respectively, in the *Sal*I-digested DNA of both of the *M. tuberculosis* strains. In *M. smegmatis*, two hybridising fragments of 4.2 and 2.2 kb, respectively, were detected. It could be concluded that there were multiple DNA fragments with homology to the known sigma^{70} genes.

Similar Southern hybridisation experiments, performed with four different clinical isolates of *M. tuberculosis*, revealed identical patterns, indicating the presence of similar genes also in other virulent isolates of *M. tuberculosis*.

EXAMPLE 2: Cloning of putative sigma^{70} homologues

2.1. Cloning of M. tuberculosis sigA

A lambda gt11 library (obtained from WHO) of the chromosomal DNA of *M. tuberculosis* Erdman strain was screened, using the 500 bp PCR probe as described above, following standard procedures. One lambda gt11 phage with a 4.7 kb *Eco*RI insert was identified and confirmed to hybridise with the PCR probe. Restriction analysis of this 4.7 kb insert revealed it to have an internal 2.2 kb *Sal*I fragment which hybridised with the PCR probe.

The 4.7 kb fragment was excised from the lambda gt 11 DNA by *Eco*RI restriction, and subcloned into the cloning vector pBR322, to obtain the recombinant plasmid pARC 8175 (Fig. 1) (NCIMB 40738).

The putative sigma^{70} homologue on the 2.2 kb *Sal*I fragment was designated *M. tuberculosis sigA*. The coding sequence of the *sigA* gene was found to have an internal *Sal*I site, which could explain the hybridisation of the 0.9 kb fragment in the Southern experiments.

2.2. *Cloning of M. tuberculosis sigB*

M. tuberculosis H37Rv DNA was restricted with *Sal*I and the DNA fragments were resolved by preparative agarose gel electrophoresis. The agarose gel piece corresponding to the 4.0 to 5.0 kb size region was cut out, and the DNA from this gel piece was extracted following standard protocols. This DNA was ligated to the cloning vector pBR329 at its *Sal*I site, and the ligated DNA was transformed into *E. coli* DH5α to obtain a sub-library. Transformants of this sub-library were identified by colony blotting, using the PCR-derived 500 bp probe, following standard protocols. Individual transformant colonies were analyzed for their plasmid profile. One of the recombinant plasmids retaining the expected plasmid size, was analyzed in detail by restriction mapping and was found to harbour the expected 4.2 kb *Sal*I DNA fragment. This plasmid with the *sigB* gene on the 4.2 kb insert was designated pARC 8176 (Fig. 2) (NCIMB 40739).

EXAMPLE 3: Nucleotide sequence of *M. tuberculosis sigA* and *sigB* genes

3.1. Nucleotide sequence of sigA

The *Eco*RV - *Eco*RI DNA fragment expected to encompass the entire *sigA* gene was subcloned into appropriate M13 vectors and both strands of the gene sequenced by the dideoxy method. The sequence obtained is shown as SEQ ID NO: 1 in the Sequence Listing. An open reading frame (ORF) of 1580 nucleotides (positions 70 to 1650 in SEQ ID NO: 1) coding for a protein of 526 amino acids was predicted from the DNA sequence. The N-terminal amino acid has been assigned tentatively based on the first GTG (initiation codon) of the ORF.

The derived amino acid sequence of the gene product SigA (SEQ ID NO: 2) showed 60% identity with the *E. coli* sigma^{70} and 70% identity with the HrdB sequence of *Streptomyces coelicolor*. The overall anatomy of the SigA sequence is compatible with that seen among sigma^{70} proteins of various organisms. This anatomy comprises a highly conserved C-terminal half, while the N-terminal half generally shows lesser homology. The two regions are linked by a stretch of amino acids which varies in length and is found to be generally unique for the protein. The SigA sequence has a similar structure, where the unconserved central stretch correspond to amino acids 270 to 306 in SEQ ID NO: 2.

The N-terminal half has limited homology to *E. coli* sigma^{70}, but shows resemblance to that of the sigma^{70} homologue HrdB of *S. coelicolor*. The highly conserved motifs of regions 3.1, 3.2, 4.1 and 4.2 of *S. coelicolor* which were proposed to be involved in DNA binding (Lonetto, M. et al. (1992) J. Bacteriol. 174, 3843-3849) are found to be nearly identical also in the *M. tuberculosis* SigA sequence. The N-terminal start of the protein has been tentatively assigned, based on homologous motifs of the *S. coelicolor* HrdB sequence.

The overall sequence similarity of the SigA and SigB amino acid sequences to known sigma^{70} sequences suggests assignment of the *M. tuberculosis* SigA to the Group I sigma^{70} proteins. However, SigA also shows distinct differences with known sigma^{70} proteins, in particular a unique and lengthy N-terminal stretch of amino acids (positions 24 to 263 in SEQ ID NO: 2), which may be essential for the recognition and initiation of transcription from promoter sequences of *M. tuberculosis*.

3.2. Nucleotide sequence of sigB

The nucleotide sequence of the *sigB* gene (SEQ ID NO: 3) encodes a protein of 323 amino acids (SEQ ID NO: 4). The N-terminal start of the protein has been tentatively identified based on the presence of the first methionine of the ORF. The ORF is thus estimated to start at position 325 and to end at 1293 in SEQ ID NO: 3. Alignment of the amino acid sequence of the *sigB* gene with other sigma^{70} proteins places the *sigB* gene into the Group I family of sigma^{70} proteins. The overall structure of the gene product SigB follows the same pattern as described for SigA. However, the SigB sequence has only 60% homology with the SigA sequence, as there are considerable differences not only within the unconserved regions of the protein, but also within the putative DNA binding regions of the sigB protein. These characteristics suggest that the SigB protein may play a distinct function in the physiology of the organism.

EXAMPLE 4: Expression of *sigA* and *sigB*

4.1. Expression of M. tuberculosis sigA gene in E. coli

The N-terminal portion of the *sigA* gene was amplified by PCR using the following primers:

Forward primer (SEQ ID NO: 7), comprising an *Nco*I site:

```
                    66nt--------------80nt
                    |                  |
5'-TT CC ATG GGG TAT GTG GCA GCG ACC-3'
5          M   G   Y   V   A   A   T
```

Reverse primer (SEQ ID NO: 8):

5'-GTA CAG GCC AGC CTC GAT CCG CTT GGC-3'

(a) A fragment of approximately 750 bp was amplified from the *sigA* gene construct pARC 8175. The amplified product was restricted with *Nco*I and *Bam*HI to obtain a 163 bp fragment.

(b) A 1400 bp DNA fragment was obtained by digestion of pARC 8175 with *Bam*HI and *Eco*RV.

(c) The expression plasmid pET 8ck, which is a derivative of pET 8c (Studier, F.W. et al. (1990) Methods Enzymol. 185, 61-89) in which the β-lactamase gene has been replaced by the gene conferring kanamycin resistance, was digested with *Nco*I and *Eco*RV and a fragment of approximately 4.2 kb was purified.

These three fragments (a), (b) and (c) were ligated by standard methods and the product was transformed into *E. coli* DH5α. Individual transformants were screened for the plasmid profile following standard protocols. The transformant was identified based on the expected plasmid size (approximately 6.35 kb) and restriction mapping of the plasmid. The recombinant plasmid harbouring the coding fragment of *sigA* was designated pARC 8171.

The plasmid pARC 8171 was transformed into the T_7 expression host *E. coli* BL21(DE3). Individual transformants were screened for the presence of the 6.35 kb plasmid and confirmed by restriction analysis. One of the

transformants was grown at 37°C and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) using standard protocols. A specific 90 kDa protein was induced on expression. Cells were harvested by low speed centrifugation and lysed by sonication in phosphate buffered saline, pH 7.4. The lysate was centrifugated at 100,000 x g to fractionate into supernatant and pellet. The majority of the 70 kDa product obtained after induction with IPTG was present in the pellet fraction, indicating that the protein formed inclusion bodies.

For purifying the induced *sigA* gene product, the cell lysate as obtained above was clarified by centrifugation at 1000 rpm in Beckman JA 21 rotor for 15 min. The clarified supernatant was layered on a 15-60% sucrose gradient and centrifugated at 100,000 x g for 60 min. The inclusion bodies sedimented as a pellet through the 60% sucrose cushion. This pellet was solubilised in 6 M guanidine hydrochloride which was removed by sequential dialysis against buffer containing decreasing concentration of guanidine hydrochloride. The dialysate was 75% enriched for the SigA protein which was purified essentially following the protocol for purification *E. coli* sigma^{70} as described by Brokhov, S. and Goldfarb, A. (1993) Protein expression and purification, vol. 4, 503-511.

4.2. Expression of M. tuberculosis sigB gene in E. coli

The *sigB* gene product was expressed and purified from inclusion bodies. The coding sequence of the *sigB* gene was amplified by PCR using the following primers:

Forward primer (SEQ ID NO: 9), comprising an *Nco*I restriction site:

5'- TTTC ATG GCC GAT GCA CCC ACA AGG GCC-3'

M A D A P T R A

Reverse primer (SEQ ID NO: 10), comprising an *Eco*RI restriction site:

5'- CTT GAA TTC AGC TGG CGT ACG ACC GCA-3'

The amplified 920 bp fragment was digested with *Eco*RI and *Nco*I and ligated to the *Eco*RI- and *Nco*I-digested pRSET B (Kroll et al. (1993) DNA and Cell Biology 12, 441). The ligation mix was transformed into *E. coli* DH5α. Individual transformants were screened for plasmid profile and restriction analysis. The recombinant plasmid having the expected plasmid profile was designated pARC 8193.

E. coli DH5α harbouring pARC 8193 was cultured in LB containing in 50 µg/ml ampicillin till an OD of 0.5, and induced with 1 mM IPTG at 37°C, following standard protocols. The induced SigB protein was obtained as inclusion bodies which were denatured and renatured following the same protocol as described for the SigA protein. The purified SigB protein was >90% homogenous and suitable for transcription assays.

DEPOSIT OF MICROORGANISMS

The following plasmids have been deposited under the Budapest Treaty at the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland, UK.

Plasmid	Accession No.	Date of deposit
pARC 8175	NCIMB 40738	15 June 1995
pARC 8176	NCIMB 40739	15 June 1995

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Astra AB
(B) STREET: Västra Mälarehamnen 9
(C) CITY: Södertälje
(E) COUNTRY: Sweden
(F) POSTAL CODE (ZIP): S-151 85
(G) TELEPHONE: +46-8-553 260 00
(H) TELEFAX: +46-8-553 288 20
(I) TELEX: 19237 astra s

(ii) TITLE OF INVENTION: New DNA Molecules

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1724 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mycobacterium tuberculosis
(B) STRAIN: Erdman strain

(vii) IMMEDIATE SOURCE:
(B) CLONE: pARC 8175

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:70..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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AACTAGCAGA CACTTTCGGT TACGCACGCC CAGACCCAAC CGGAAGTGAG TAACGACCGA      60

AGGGTGTAT GTG GCA GCG ACC AAA GCA AGC ACG GCG ACC GAT GAG CCG          108
          Val Ala Ala Thr Lys Ala Ser Thr Ala Thr Asp Glu Pro
            1               5                  10

GTA AAA CGC ACC GCC ACC AAG TCG CCC GCG GCT TCC GCG TCC GGG GCC        156
Val Lys Arg Thr Ala Thr Lys Ser Pro Ala Ala Ser Ala Ser Gly Ala
     15                  20                  25

AAG ACC GGC GCC AAG CGA ACA GCG GCG AAG TCC GCT AGT GGC TCC CCA        204
Lys Thr Gly Ala Lys Arg Thr Ala Ala Lys Ser Ala Ser Gly Ser Pro
 30                  35                  40                  45

CCC GCG AAG CGG GCT ACC AAG CCC GCG GCC CGG TCC GTC AAG CCC GCC        252
Pro Ala Lys Arg Ala Thr Lys Pro Ala Ala Arg Ser Val Lys Pro Ala
                50                  55                  60
```

```
TCG GCA CCC CAG GAC ACT ACG ACC AGC ACC ATC CCG AAA AGG AAG ACC        300
Ser Ala Pro Gln Asp Thr Thr Thr Ser Thr Ile Pro Lys Arg Lys Thr
               65                  70                  75

CGC GCC GCG GCC AAA TCC GCC GCC GCG AAG GCA CCG TCG GCC CGC GGC        348
Arg Ala Ala Ala Lys Ser Ala Ala Ala Lys Ala Pro Ser Ala Arg Gly
         80                  85                  90

CAC GCG ACC AAG CCA CGG GCG CCC AAG GAT GCC CAG CAC GAA GCC GCA        396
His Ala Thr Lys Pro Arg Ala Pro Lys Asp Ala Gln His Glu Ala Ala
     95                  100                 105

ACG GAT CCC GAG GAC GCC CTG GAC TCC GTC GAG GAG CTC GAC GCT GAA        444
Thr Asp Pro Glu Asp Ala Leu Asp Ser Val Glu Glu Leu Asp Ala Glu
110                 115                 120                 125

CCA GAC CTC GAC GTC GAG CCC GGC GAG GAC CTC GAC CTT GAC GCC GCC        492
Pro Asp Leu Asp Val Glu Pro Gly Glu Asp Leu Asp Leu Asp Ala Ala
                130                 135                 140

GAC CTC AAC CTC GAT GAC CTC GAG GAC GAC GTG GCG CCG GAC GCC GAC        540
Asp Leu Asn Leu Asp Asp Leu Glu Asp Asp Val Ala Pro Asp Ala Asp
            145                 150                 155

GAC GAC CTC GAC TCG GGC GAC GAC GAA GAC CAC GAA GAC CTC GAA GCT        588
Asp Asp Leu Asp Ser Gly Asp Asp Glu Asp His Glu Asp Leu Glu Ala
        160                 165                 170

GAG GCG GCC GTC GCG CCC GGC CAG ACC GCC GAT GAC GAC GAG GAG ATC        636
Glu Ala Ala Val Ala Pro Gly Gln Thr Ala Asp Asp Asp Glu Glu Ile
    175                 180                 185

GCT GAA CCC ACC GAA AAG GAC AAG GCC TCC GGT GAT TTC GTC TGG GAT        684
Ala Glu Pro Thr Glu Lys Asp Lys Ala Ser Gly Asp Phe Val Trp Asp
190                 195                 200                 205

GAA GAC GAG TCG GAG GCC CTG CGT CAA GCA CGC AAG GAC GCC GAA CTC        732
Glu Asp Glu Ser Glu Ala Leu Arg Gln Ala Arg Lys Asp Ala Glu Leu
                210                 215                 220

ACC GCA TCC GCC GAC TCG GTT CGC GCC TAC CTC AAA CAG ATC GGC AAG        780
Thr Ala Ser Ala Asp Ser Val Arg Ala Tyr Leu Lys Gln Ile Gly Lys
            225                 230                 235

GTA GCG CTG CTC AAC GCC GAG GAA GAG GTC GAG CTA GCC AAG CGG ATC        828
Val Ala Leu Leu Asn Ala Glu Glu Glu Val Glu Leu Ala Lys Arg Ile
        240                 245                 250

GAG GCT GGC CTG TAC GCC ACG CAG CTG ATG ACC GAG CTT AGC GAG CGC        876
Glu Ala Gly Leu Tyr Ala Thr Gln Leu Met Thr Glu Leu Ser Glu Arg
    255                 260                 265

GGC GAA AAG CTG CCT GCC GCC CAG CGC CGC GAC ATG ATG TGG ATC TGC        924
Gly Glu Lys Leu Pro Ala Ala Gln Arg Arg Asp Met Met Trp Ile Cys
270                 275                 280                 285

CGC GAC GGC GAT CGC GCG AAA AAC CAT CTG CTG GAA GCC AAC CTG CGC        972
Arg Asp Gly Asp Arg Ala Lys Asn His Leu Leu Glu Ala Asn Leu Arg
                290                 295                 300

CTG GTG GTT TCG CTA GCC AAG CGC TAC ACC GGC CGG GGC ATG GCG TTT       1020
Leu Val Val Ser Leu Ala Lys Arg Tyr Thr Gly Arg Gly Met Ala Phe
            305                 310                 315

CTC GAC CTG ATC CAG GAA GGC AAC CTG GGG CTG ATC CGC GCG GTG GAG       1068
Leu Asp Leu Ile Gln Glu Gly Asn Leu Gly Leu Ile Arg Ala Val Glu
        320                 325                 330
```

```
AAG TTC GAC TAC ACC AAG GGG TAC AAG TTC TCC ACC TAC GCT ACG TGG      1116
Lys Phe Asp Tyr Thr Lys Gly Tyr Lys Phe Ser Thr Tyr Ala Thr Trp
    335                 340                 345

TGG ATT CGC CAG GCC ATC ACC CGC GCC ATG GCC GAC CAG GCC CGC ACC      1164
Trp Ile Arg Gln Ala Ile Thr Arg Ala Met Ala Asp Gln Ala Arg Thr
350                 355                 360                 365

ATC CGC ATC CCG GTG CAC ATG GTC GAG GTG ATC AAC AAG CTG GGC CGC      1212
Ile Arg Ile Pro Val His Met Val Glu Val Ile Asn Lys Leu Gly Arg
                370                 375                 380

ATT CAA CGC GAG CTG CTG CAG GAC CTG GGC CGC GAG CCC ACG CCC GAG      1260
Ile Gln Arg Glu Leu Leu Gln Asp Leu Gly Arg Glu Pro Thr Pro Glu
            385                 390                 395

GAG CTG GCC AAA GAG ATG GAC ATC ACC CCG GAG AAG GTG CTG GAA ATC      1308
Glu Leu Ala Lys Glu Met Asp Ile Thr Pro Glu Lys Val Leu Glu Ile
        400                 405                 410

CAG CAA TAC GCC CGC GAG CCG ATC TCG TTG GAC CAG ACC ATC GGC GAC      1356
Gln Gln Tyr Ala Arg Glu Pro Ile Ser Leu Asp Gln Thr Ile Gly Asp
    415                 420                 425

GAG GGC GAC AGC CAG CTT GGC GAT TTC ATC GAA GAC AGC GAG GCG GTG      1404
Glu Gly Asp Ser Gln Leu Gly Asp Phe Ile Glu Asp Ser Glu Ala Val
430                 435                 440                 445

GTG GCC GTC GAC GCG GTG TCC TTC ACT TTG CTG CAG GAT CAA CTG CAG      1452
Val Ala Val Asp Ala Val Ser Phe Thr Leu Leu Gln Asp Gln Leu Gln
                450                 455                 460

TCG GTG CTG GAC ACG CTC TCC GAG CGT GAG GCG GGC GTG GTG CGG CTA      1500
Ser Val Leu Asp Thr Leu Ser Glu Arg Glu Ala Gly Val Val Arg Leu
            465                 470                 475

CGC TTC GGC CTT ACC GAC GGC CAG CCG CGC ACC CTT GAC GAG ATC GGC      1548
Arg Phe Gly Leu Thr Asp Gly Gln Pro Arg Thr Leu Asp Glu Ile Gly
        480                 485                 490

CAG GTC TAC GGC GTG ACC CGG GAA CGC ATC CGC CAG ATC GAA TCC AAG      1596
Gln Val Tyr Gly Val Thr Arg Glu Arg Ile Arg Gln Ile Glu Ser Lys
    495                 500                 505

ACT ATG TCG AAG TTG CGC CAT CCG AGC CGC TCA CAG GTC CTG CGC GAC      1644
Thr Met Ser Lys Leu Arg His Pro Ser Arg Ser Gln Val Leu Arg Asp
510                 515                 520                 525

TAC CTG GAC TGAGAGCGCC CGCCGAGGCG ACCAACGTAG CACGTGAGCC              1693
Tyr Leu Asp

CCCAGCAGCT AGCCGCACCA TGGTCTCGTC C                                   1724
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 528 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Val Ala Ala Thr Lys Ala Ser Thr Ala Thr Asp Glu Pro Val Lys Arg
 1               5                  10                  15
```

```
Thr Ala Thr Lys Ser Pro Ala Ala Ser Ala Ser Gly Ala Lys Thr Gly
                20                  25                  30

Ala Lys Arg Thr Ala Ala Lys Ser Ala Ser Gly Ser Pro Pro Ala Lys
            35                  40                  45

Arg Ala Thr Lys Pro Ala Ala Arg Ser Val Lys Pro Ala Ser Ala Pro
        50                  55                  60

Gln Asp Thr Thr Thr Ser Thr Ile Pro Lys Arg Lys Thr Arg Ala Ala
65                  70                  75                  80

Ala Lys Ser Ala Ala Ala Lys Ala Pro Ser Ala Arg Gly His Ala Thr
                85                  90                  95

Lys Pro Arg Ala Pro Lys Asp Ala Gln His Glu Ala Ala Thr Asp Pro
            100                 105                 110

Glu Asp Ala Leu Asp Ser Val Glu Glu Leu Asp Ala Glu Pro Asp Leu
        115                 120                 125

Asp Val Glu Pro Gly Glu Asp Leu Asp Leu Asp Ala Ala Asp Leu Asn
    130                 135                 140

Leu Asp Asp Leu Glu Asp Asp Val Ala Pro Asp Ala Asp Asp Asp Leu
145                 150                 155                 160

Asp Ser Gly Asp Asp Glu Asp His Glu Asp Leu Glu Ala Glu Ala Ala
                165                 170                 175

Val Ala Pro Gly Gln Thr Ala Asp Asp Asp Glu Glu Ile Ala Glu Pro
            180                 185                 190

Thr Glu Lys Asp Lys Ala Ser Gly Asp Phe Val Trp Asp Glu Asp Glu
        195                 200                 205

Ser Glu Ala Leu Arg Gln Ala Arg Lys Asp Ala Glu Leu Thr Ala Ser
    210                 215                 220

Ala Asp Ser Val Arg Ala Tyr Leu Lys Gln Ile Gly Lys Val Ala Leu
225                 230                 235                 240

Leu Asn Ala Glu Glu Glu Val Glu Leu Ala Lys Arg Ile Glu Ala Gly
                245                 250                 255

Leu Tyr Ala Thr Gln Leu Met Thr Glu Leu Ser Glu Arg Gly Glu Lys
            260                 265                 270

Leu Pro Ala Ala Gln Arg Arg Asp Met Met Trp Ile Cys Arg Asp Gly
        275                 280                 285

Asp Arg Ala Lys Asn His Leu Leu Glu Ala Asn Leu Arg Leu Val Val
    290                 295                 300

Ser Leu Ala Lys Arg Tyr Thr Gly Arg Gly Met Ala Phe Leu Asp Leu
305                 310                 315                 320

Ile Gln Glu Gly Asn Leu Gly Leu Ile Arg Ala Val Glu Lys Phe Asp
                325                 330                 335

Tyr Thr Lys Gly Tyr Lys Phe Ser Thr Tyr Ala Thr Trp Trp Ile Arg
            340                 345                 350

Gln Ala Ile Thr Arg Ala Met Ala Asp Gln Ala Arg Thr Ile Arg Ile
        355                 360                 365

Pro Val His Met Val Glu Val Ile Asn Lys Leu Gly Arg Ile Gln Arg
    370                 375                 380
```

```
Glu Leu Leu Gln Asp Leu Gly Arg Glu Pro Thr Pro Glu Glu Leu Ala
385                 390                 395                 400

Lys Glu Met Asp Ile Thr Pro Glu Lys Val Leu Glu Ile Gln Gln Tyr
                405                 410                 415

Ala Arg Glu Pro Ile Ser Leu Asp Gln Thr Ile Gly Asp Glu Gly Asp
            420                 425                 430

Ser Gln Leu Gly Asp Phe Ile Glu Asp Ser Glu Ala Val Val Ala Val
        435                 440                 445

Asp Ala Val Ser Phe Thr Leu Leu Gln Asp Gln Leu Gln Ser Val Leu
    450                 455                 460

Asp Thr Leu Ser Glu Arg Glu Ala Gly Val Val Arg Leu Arg Phe Gly
465                 470                 475                 480

Leu Thr Asp Gly Gln Pro Arg Thr Leu Asp Glu Ile Gly Gln Val Tyr
                485                 490                 495

Gly Val Thr Arg Glu Arg Ile Arg Gln Ile Glu Ser Lys Thr Met Ser
            500                 505                 510

Lys Leu Arg His Pro Ser Arg Ser Gln Val Leu Arg Asp Tyr Leu Asp
        515                 520                 525
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1508 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mycobacterium tuberculosis
(C) INDIVIDUAL ISOLATE: atcc27294

(vii) IMMEDIATE SOURCE:
(B) CLONE: pARC 8176

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:325..1293

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
ACCAGCCCGA CGACCGACGA ACCCCGCCGC TTCGACGTGC CCAGCCGGCG CATCCCGCTG      60

TTCCCGACCG CGAACGGCCC GCACTCGAGC CGACGGCGAC AGCCGGCAAG AAGCGGTCAG     120

CCCGCGGGGA TTCGCCGACC ACGGTTAGCC GTCTGTTGGC CGGCGTTCCG GGTTGTCGCC     180

ACTGGCCACA CTTCTCAGGA CTTTCTCAGG TCTTCGGCAG ATTCCTGCAC GTCACAGGGC     240

GTCAGATCAC TGCTGGGTGG GAACTCAAAG TCCGGCTTTG TCGTTAAACC CTGACAGTGC     300

AAGCCGATCG GGGAACGGCT CGCT ATG GCC GAT GCA CCC ACA AGG GCC ACC         351
                          Met Ala Asp Ala Pro Thr Arg Ala Thr
                              530                 535
```

```
ACA AGC CGG GTT GAC ACA GAT CTG GAT GCT CAA AGC CCC GCG GCG GAC      399
Thr Ser Arg Val Asp Thr Asp Leu Asp Ala Gln Ser Pro Ala Ala Asp
        540                 545                 550

CTC GTG CGC GTC TAT CTG AAC GGC ATC GGC AAG ACG GCG TTG CTC AAC      447
Leu Val Arg Val Tyr Leu Asn Gly Ile Gly Lys Thr Ala Leu Leu Asn
    555                 560                 565

GCG GCG GAT GAA GTC GAA CTG GCC AAG CGC ATA GAA GCC GGG TTG TAT      495
Ala Ala Asp Glu Val Glu Leu Ala Lys Arg Ile Glu Ala Gly Leu Tyr
570                 575                 580                 585

GCC GAG CAT CTG CTG GAA ACC CGG AAG CGC CTC GGC GAG AAC CGA AAA      543
Ala Glu His Leu Leu Glu Thr Arg Lys Arg Leu Gly Glu Asn Arg Lys
                590                 595                 600

CGC GAC CTG GCG GCC GTG GTG CGT GAT GGC GAG GCC GCC CGC CGC CAC      591
Arg Asp Leu Ala Ala Val Val Arg Asp Gly Glu Ala Ala Arg Arg His
            605                 610                 615

CTG CTG GAA GCA AAC CTG CGG CTG GTG GTA TCG CTG GCC AAG CGC TAC      639
Leu Leu Glu Ala Asn Leu Arg Leu Val Val Ser Leu Ala Lys Arg Tyr
        620                 625                 630

ACG GGT CGG GGC ATG CCG TTG CTG GAC CTC ATC CAG GAG GGC AAC CTG      687
Thr Gly Arg Gly Met Pro Leu Leu Asp Leu Ile Gln Glu Gly Asn Leu
    635                 640                 645

GGT CTG ATC CGA GCG ATG GAG AAG TTC GAC TAC ACA AAG GGA TTC AAG      735
Gly Leu Ile Arg Ala Met Glu Lys Phe Asp Tyr Thr Lys Gly Phe Lys
650                 655                 660                 665

TTC TCA ACG TAT GCC ACG TGG TGG ATC CGC CAG GCC ATC ACC CGC GGA      783
Phe Ser Thr Tyr Ala Thr Trp Trp Ile Arg Gln Ala Ile Thr Arg Gly
                670                 675                 680

ATG GCC GAC CAG AGC CGC ACC ATC CGC CTG CCC GTA CAC CTG GTT GAG      831
Met Ala Asp Gln Ser Arg Thr Ile Arg Leu Pro Val His Leu Val Glu
            685                 690                 695

CAG GTC AAC AAG CTG GCG CGG ATC AAG CGG GAG ATG CAC CAG CAT CTG      879
Gln Val Asn Lys Leu Ala Arg Ile Lys Arg Glu Met His Gln His Leu
        700                 705                 710

GGT CGC GAA CGC ACC GAT GAG GAG CTC GCC GCC GAA TCC GGC ATT CCA      927
Gly Arg Glu Arg Thr Asp Glu Glu Leu Ala Ala Glu Ser Gly Ile Pro
    715                 720                 725

ATC GAC AAG ATC AAC GAC CTG CTG GAA CAC AGT CGC GAC CCG GTG AGT      975
Ile Asp Lys Ile Asn Asp Leu Leu Glu His Ser Arg Asp Pro Val Ser
730                 735                 740                 745

CTG GAT ATG CCG GTC GGC TCC GAG GAG GAG GCC CCT TTG GGC GAT TTC     1023
Leu Asp Met Pro Val Gly Ser Glu Glu Glu Ala Pro Leu Gly Asp Phe
                750                 755                 760

ATC GAG GAC GCC GAA GCC ATG TCC GCG GAG AAC GCG GTC ATC GCC GAA     1071
Ile Glu Asp Ala Glu Ala Met Ser Ala Glu Asn Ala Val Ile Ala Glu
            765                 770                 775

CTG TTA CAC ACC GAC ATC CGC AGC GTG CTG GCC ACT CTC GAC GAG CGT     1119
Leu Leu His Thr Asp Ile Arg Ser Val Leu Ala Thr Leu Asp Glu Arg
        780                 785                 790

GAC GAC CAG GTG ATC CGG CTG CGC TTC GGC CTG GAT GAC GGC CAA CCA     1167
Asp Asp Gln Val Ile Arg Leu Arg Phe Gly Leu Asp Asp Gly Gln Pro
    795                 800                 805
```

```
CGC ACC CTG GAT CAA ATC GGC AAA CTA TTC GGG CTG TCC CGT GAG CGG      1215
Arg Thr Leu Asp Gln Ile Gly Lys Leu Phe Gly Leu Ser Arg Glu Arg
810                 815                 820                 825

GTT CGT CAG ATC GAG CGC GAC GTG ATG AGT AAG CTG CGG CAC GGT GAG      1263
Val Arg Gln Ile Glu Arg Asp Val Met Ser Lys Leu Arg His Gly Glu
                830                 835                 840

CGG GCG GAT CGG CTG CGG TCG TAC GCC AGC TGAAGCTGGA CATCCTGAGC        1313
Arg Ala Asp Arg Leu Arg Ser Tyr Ala Ser
            845                 850

CAGGTAGCAG ACGGTATGCC CGCCGCGCCA GCATAGCCTG CGGTGGGGCG GCGGGCAACC    1373

ATTTTCGCAG CTGGCCAAGT GTAGACTCAG CTGCAATGGA GGGTGCTGAA TGAACGAGTT    1433

GGTTGATACC ACCGAGATGT ACCTGCGGAC CATCTACGAC CTCGAGGAAG AGGGCGTGAC    1493

GCACTGCGTG CCGGA                                                    1508
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 323 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Ala Asp Ala Pro Thr Arg Ala Thr Thr Ser Arg Val Asp Thr Asp
 1               5                  10                  15

Leu Asp Ala Gln Ser Pro Ala Ala Asp Leu Val Arg Val Tyr Leu Asn
            20                  25                  30

Gly Ile Gly Lys Thr Ala Leu Leu Asn Ala Ala Asp Glu Val Glu Leu
        35                  40                  45

Ala Lys Arg Ile Glu Ala Gly Leu Tyr Ala Glu His Leu Leu Glu Thr
    50                  55                  60

Arg Lys Arg Leu Gly Glu Asn Arg Lys Arg Asp Leu Ala Ala Val Val
65                  70                  75                  80

Arg Asp Gly Glu Ala Ala Arg Arg His Leu Leu Glu Ala Asn Leu Arg
                85                  90                  95

Leu Val Val Ser Leu Ala Lys Arg Tyr Thr Gly Arg Gly Met Pro Leu
            100                 105                 110

Leu Asp Leu Ile Gln Glu Gly Asn Leu Gly Leu Ile Arg Ala Met Glu
        115                 120                 125

Lys Phe Asp Tyr Thr Lys Gly Phe Lys Phe Ser Thr Tyr Ala Thr Trp
    130                 135                 140

Trp Ile Arg Gln Ala Ile Thr Arg Gly Met Ala Asp Gln Ser Arg Thr
145                 150                 155                 160

Ile Arg Leu Pro Val His Leu Val Glu Gln Val Asn Lys Leu Ala Arg
                165                 170                 175

Ile Lys Arg Glu Met His Gln His Leu Gly Arg Glu Arg Thr Asp Glu
            180                 185                 190
```

```
Glu Leu Ala Ala Glu Ser Gly Ile Pro Ile Asp Lys Ile Asn Asp Leu
        195                 200                 205

Leu Glu His Ser Arg Asp Pro Val Ser Leu Asp Met Pro Val Gly Ser
    210                 215                 220

Glu Glu Glu Ala Pro Leu Gly Asp Phe Ile Glu Asp Ala Glu Ala Met
225                 230                 235                 240

Ser Ala Glu Asn Ala Val Ile Ala Glu Leu Leu His Thr Asp Ile Arg
                245                 250                 255

Ser Val Leu Ala Thr Leu Asp Glu Arg Asp Asp Gln Val Ile Arg Leu
            260                 265                 270

Arg Phe Gly Leu Asp Asp Gly Gln Pro Arg Thr Leu Asp Gln Ile Gly
        275                 280                 285

Lys Leu Phe Gly Leu Ser Arg Glu Arg Val Arg Gln Ile Glu Arg Asp
    290                 295                 300

Val Met Ser Lys Leu Arg His Gly Glu Arg Ala Asp Arg Leu Arg Ser
305                 310                 315                 320

Tyr Ala Ser
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AAGTTCAGCA CSTACGCSAC STGGTGGATC 30

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTTSGCCTCG ATCTGSCGGA TSCGCTC 27

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TTCCATGGGG TATGTGGCAG CGACC 25

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTACAGGCCA GCCTCGATCC GCTTGGC 27

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTTCATGGCC GATGCACCCA CAAGGGCC 28

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CTTGAATTCA GCTGGCGTAC GACCGCA 27

CLAIMS

1. An isolated polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* RNA polymerase, or a functionally equivalent modified form thereof.

2. A polypeptide according to claim 1 which amino acid sequence is identical to, or substantially similar to, SEQ ID NO: 2 or 4 in the Sequence Listing.

3. An isolated nucleic acid molecule which has a nucleotide sequence coding for a polypeptide according to claim 1 or 2.

4. An isolated nucleic acid molecule selected from:
(a) DNA molecules comprising a nucleotide sequence as shown in SEQ ID NO: 1 or SEQ ID NO: 3 encoding a Group I sigma subunit of *Mycobacterium tuberculosis* RNA polymerase;
(b) nucleic acid molecules comprising a nucleotide sequence capable of hybridizing to a nucleotide sequence complementary the polypeptide coding region of a DNA molecule as defined in (a) and which codes for a polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* or a functionally equivalent modified form thereof; and
(c) nucleic acid molecules comprising a nucleic acid sequence which is degenerate, as a result of the genetic code, to a nucleotide sequence as defined in (a) or (b) and which codes for a polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* or a functionally equivalent modified form thereof.

5. A vector which comprises a nucleic acid molecule according to claim 3 or 4.

6. A vector according to claim 5 which is the plasmid vector pARC 8175 (NCIMB 40738) or pARC 8176 (NCIMB 40739).

7. A vector according to claim 5 which is an expression vector capable of mediating the expression of a polypeptide according to claim 1 or 2.

8. A host cell harbouring a vector according to any one of claims 5 to 7.

9. A process for production of a polypeptide according to claim 1 or 2 which comprises culturing a host cell according to claim 8 transformed with an expression vector according to claim 7 under conditions whereby said polypeptide is produced and recovering said polypeptide.

10. A method of assaying for compounds which have the ability to inhibit the association of a sigma subunit with a *Mycobacterium tuberculosis* core RNA polymerase, said method comprising (i) contacting a compound to be tested for said inhibition ability with a polypeptide according to claim 1 or claim 2 and a *Mycobacterium tuberculosis* core RNA polymerase; and (ii) detecting whether the said polypeptide associates with the said core RNA polymerase to form RNA polymerase holoenzyme.

11. A method according to claim 10 wherein polypeptides which are associated to core RNA polymerase and / or polypeptides which are not associated to core RNA polymerase are detected by chromatography such as gel filtration.

12. A method according to claim 10 wherein RNA polymerase holoenzyme is detected by immunoprecipitation, using an antibody binding to RNA polymerase holoenzyme.

13. A method of assaying for compounds which have the ability to inhibit sigma subunit-dependent transcription by a *Mycobacterium tuberculosis* RNA polymerase, said method comprising (i) contacting a compound to be tested for said inhibition ability with a polypeptide according to claim 1 or claim 2, a *Mycobacterium tuberculosis* core RNA polymerase, and a DNA having a coding sequence operably-linked to a promoter sequence capable of recognition by said core RNA polymerase when bound to said polypeptide, said contacting being carried out under conditions suitable for transcription of said coding sequence when *Mycobacterium tuberculosis* RNA polymerase is bound to said promoter; and (ii) detecting formation of mRNA corresponding to said coding sequence.

14. A method of determining the protein structure of a *Mycobacterium tuberculosis* RNA polymerase sigma subunit, characterised in that a polypeptide according to claim 1 or claim 2 is utilized in X-ray crystallography.

15. A polypeptide according to claim 1 substantially as described in the Examples.

16. An isolated nucleic acid according to claim 3 or 4 substantially as described in the Examples.

17. A vector according to claim 5 substantially as described in the Examples.

18. A host cell according to claim 8 substantially as described in the Examples.

The Patent Office

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Application No: GB 9603860.9 **Examiner:** Dr. Nicola Curtis
Claims searched: 1-18 **Date of search:** 30 April 1996

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.O): C3H (HB7P; HC2)

Int Cl (Ed.6): C07K 14/35

Other: ONLINE: WPI; BIOTECH/DIALOG; CAS ONLINE

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
E,X	WO 95/17511 A2 (Agresearch New Zealand Pastoral Agriculture Research Institute) (See Fig. 9A)	3-5
P,X	PROC. NATL. ACAD. SCI., Vol. 92, August 1995, Collins et al., "Mutation of the principal sigma factor causes loss of virulence in a strain of the *Mycobacterium tuberculosis* complex", pages 8036-8040 (See Fig. 4)	3-5
P,X	GENE, Vol. 165, 1995, Doukhan et al., "Genomic organization of the mycobacterial sigma gene cluster", pages 67-70. (See "Mycobacterial sigma genes")	1-8
X	JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT 0 (19B). 1995. 73, Balganesh et al. "Sigma factors of M. tuberculosis RNA polymerase". (See abstract)	1-8

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.